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A light and electron microscopic study of proliferation and maturation of fibrous astrocytes in the optic nerve of the human embryo

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INTRODUCTION

The criteria for electron microscopic identification of adult astrocytes are now well established (Maxwell & Kruger, 1965; Mori & Leblond, 1969; Peters, Palay & Webster, 1970) and ultrastructural studies of developing astrocytes have been carried out in the rat optic nerve by Vaughn & Peters (1967) and Peters & Vaughn (1967). Astrocytic development, as part of general glial development, has also been described in the rat by Caley & Maxwell (1968) and Vaughn (1969), in the cat by Blunt, Baldwin & Wendell-Smith (1972), in the monkey by Phillips (1973) and in the mouse by Sturrock (1974).

The optic nerve is suitable for the study of developing astrocytes as the immature nerve contains a very high proportion of these cells (Vaughn, 1969; Blunt, Baldwin & Wendell-Smith, 1972). In the present work, development of astrocytes was studied in optic nerves from 8 to 18 week human embryos.

MATERIALS AND METHODS

The materials used in this study consisted of perfusion-fixed optic nerves removed from human embryos of 28, 50, 75, 105, 120 and 165 mm crown–rump (C.R.) length, corresponding approximately to post-conceptional ages of 8, 10, 12, 14, 15 and 18 weeks respectively.

The embryos were obtained from therapeutic abortions carried out by hysterotomy. None of the abortions was carried out for reasons of suspected fetal abnormality (e.g. on mothers with rubella). Immediately after removal from the uterus each embryo, complete with membranes, was taken to an adjacent side-room and perfused via the ascending aorta with heparinised physiological saline at room temperature for one to two minutes, followed by an ice-cold solution of 2% paraformaldehyde and 3% glutaraldehyde in 0·1 M Sorensen phosphate buffer (pH 7·4) with 4% sucrose. 100 drops of 1% calcium chloride had been added to each litre of solution. Perfusion was continued for twenty minutes at a constant pressure of 100 mmHg, using a 21 gauge needle. The embryo was then decapitated, the skull was opened, and the head placed in fixative at 4 °C for four hours. At the end of this time the optic nerves, from the chiasma to the junction of the nerve with the retina, were removed and rinsed for one hour in cacodylate buffer before being post-

fixed in 1% buffered osmium tetroxide, dehydrated and embedded in Araldite. After embedding, each nerve was cut half way along its length.

At each age a series of 1 μm coronal sections was obtained from the middle of the nerve using a Spencer microtome. Every tenth section was placed on a slide and stained with 0·5% toluidine blue. Ten sections were examined at each age using an oil immersion lens. Each mitotic figure found was recorded. Every second section was photographed using a Zeiss photomicroscope. Prints were made at a magnification of $\times 400$ and all the nuclei, excluding those of endothelial cells, were counted. The percentage of mitotic figures and the average number of cells per section at each age were recorded.

The area of each section was estimated by measuring the area of the photomicrographs with a planimeter and the number of cells per unit area (1000 μm^2) at each age calculated.

Changes in vascularization of the optic nerve with development were calculated by placing an acetate sheet, with points marked on it at 1 cm intervals, over the photomicrographs. Each point which lay over the nerve was counted and each point which lay within a blood vessel or on the wall of a blood vessel was noted. The percentage of points overlying blood vessels was estimated, and the correlation coefficient of percentage vascularity and cells per section was calculated.

Ultrathin, complete transverse sections of the optic nerve, cut with a Reichert ultramicrotome were mounted on copper grids, stained with uranyl acetate and lead citrate and examined in an AEI 801 electron microscope. The ultrathin sections for electron microscopy alternated with the 1 μm sections for light microscopy.

RESULTS

Light microscopy

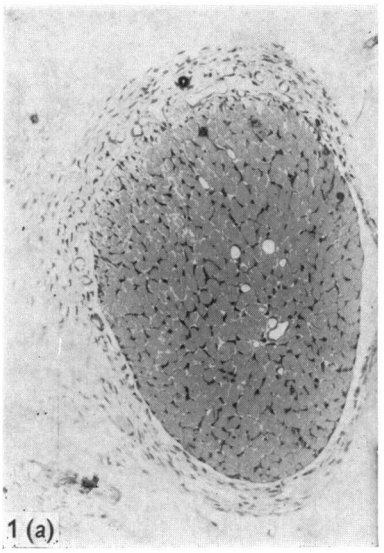
As the optic nerve matured the most striking features were the increase in vascularity (Fig. 1) and the increasing fasciculation of the nerve by cell processes (Fig. 2). At 8 weeks post-conception the nerve was split into discrete bundles of fibres by rings of cells and cell processes. With increasing age these bundles were further and further sub-divided by more and more fine processes (Fig. 2).

The number of cells per section remained virtually unchanged from 8 to 10 weeks post-conception but increased steadily thereafter. The number of cells per unit area varied from 421 cells/100 μm^2 at 12 weeks to 220 cells/1000 μm^2 at 15 weeks, with a mean value of 318 ± 67.

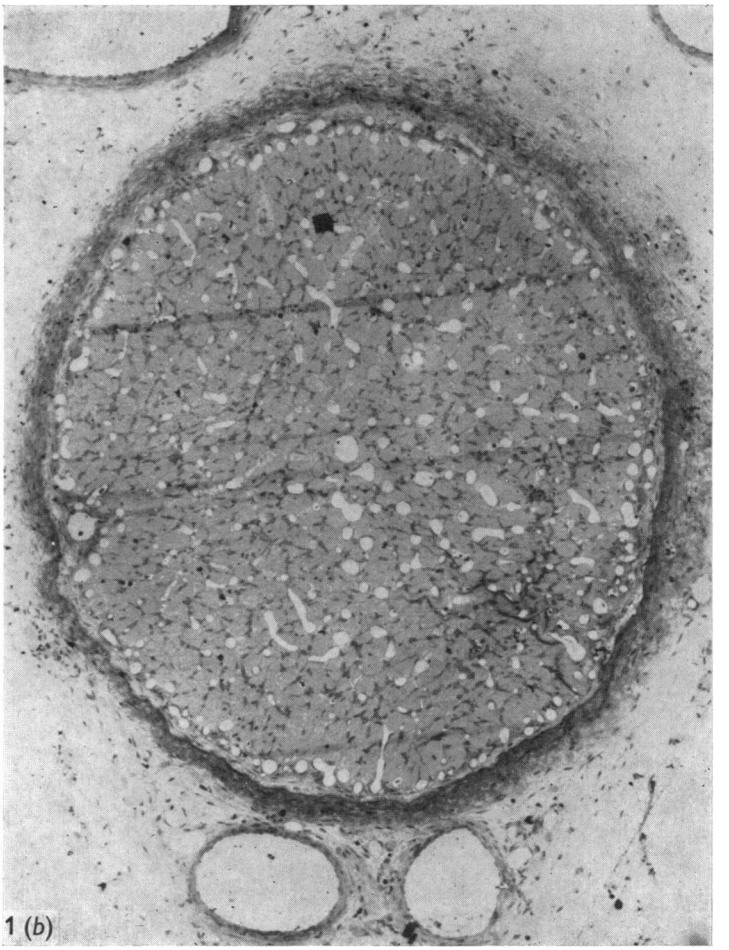
The percentage area taken up by blood vessels changed very little between 8 and 12 week post-conception, but increased from 5·5% at 12 weeks to 15·5% at 14 weeks, to 18·3% at 15 weeks and to 20·8% at 18 weeks (Table 1).

Fig. 1. This figure shows coronal sections of optic nerve from (*a*) an 8 week and (*b*) a 15 week post-conception human embryo. Note the great increase in vascularity at 15 weeks. In estimating the degree of vascularization the peripheral ring of capillaries was excluded. Toluidine blue. $\times 100$.

Fig. 2. Toluidine blue stained light microscopic sections from (*a*) 8, (*b*) 12 and (*c*) 15 week transversely sectioned optic nerves. Note the increase in fasciculation with age. (*b*) and (*c*) both contain mitotic figures. The nerve fibres appear to be more loosely packed at 8 weeks. $\times 1800$.



1 (*a*)



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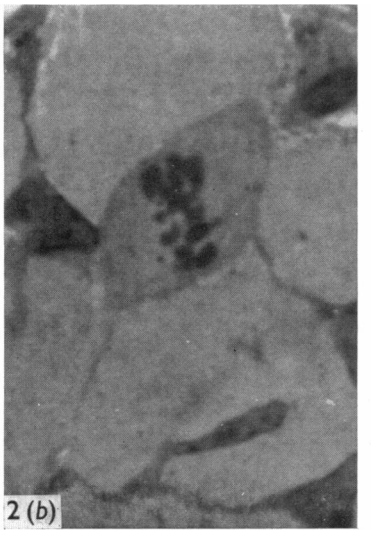
2 (*a*)



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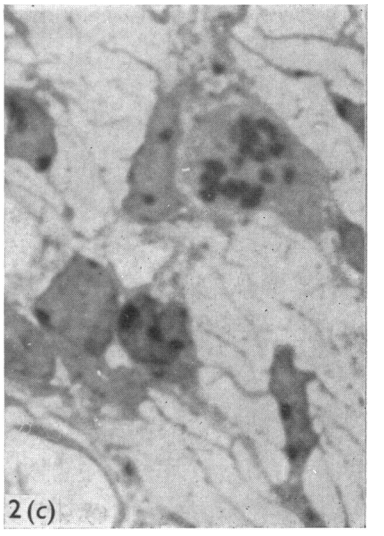


2 (*c*)

Table 1. *Mean number of cells and mitotic figures per section, mitotic index, cell density and percentage vascularisation of the developing human optic nerve*

Age (weeks post-conception)	Mean number of cells per section	Mean number of mitotic figures per section	Mitotic index (%)	Cell density per 1000 μm^2	Percentage vascularisation
8	439±26	3·0±0·8	0·68	318	4·9
10	376±47	2·4±1·4	0·64	292	4·5
12	680±65	2·8±1·6	0·41	421	5·5
14	920±77	2·7±2·6	0·29	300	15·5
15	1186±88	3·4±2·6	0·33	220	18·3
18	1847±78	1·8±1·5	0·10	355	20·8

When the number of cells per section and the percentage vascularization with increasing age were compared, a close correlation ($r = 0{\cdot}92$) was found between the results. With six degrees of freedom this correlation was significant ($p < 0{\cdot}01$).

Mitotic figures were present at all ages, and all phases of mitosis were found. The mean number of mitotic figures per section showed little variation with age (Table 1) until 18 weeks post-conception when the mean number per section fell from 3·4 to 1·8. The mitotic index showed a fall throughout the study, except between 14 and 15 weeks (Table 1). A few pyknotic cells were observed at 8, 12, 15 and 18 weeks but there seemed to be no definite pattern.

Electron microscopy

Although fasciculation of the optic nerve was observed electron microscopically at all ages, the axons appeared to be much more loosely packed in younger embryos (contrast Figs. 3 and 4 with Figs. 9 and 10).

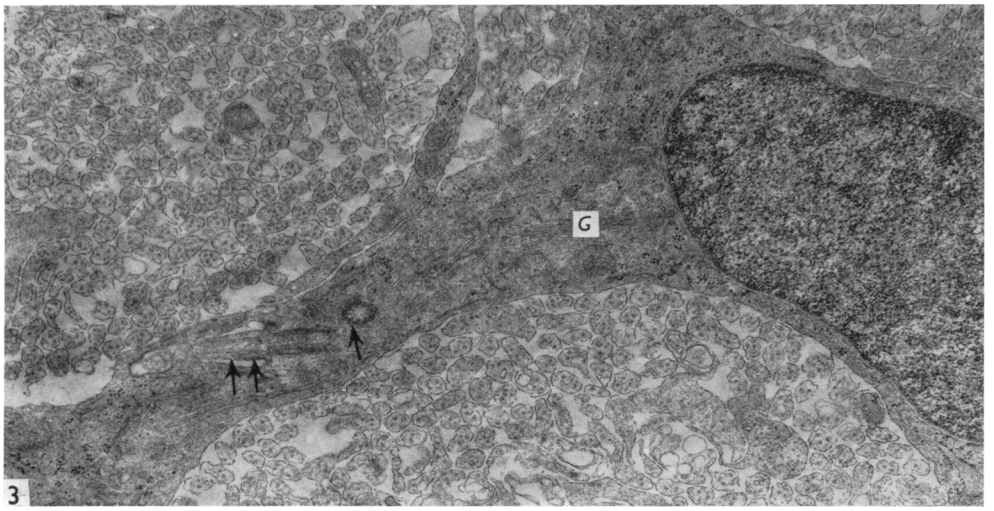
Glioblasts. These cells were found at 8, 10 and 12 weeks post-conception. At 8 weeks all the cells present were of this type and they still formed the majority of cells at 10 weeks. Only a small proportion of the total population at 12 weeks was composed of glioblasts.

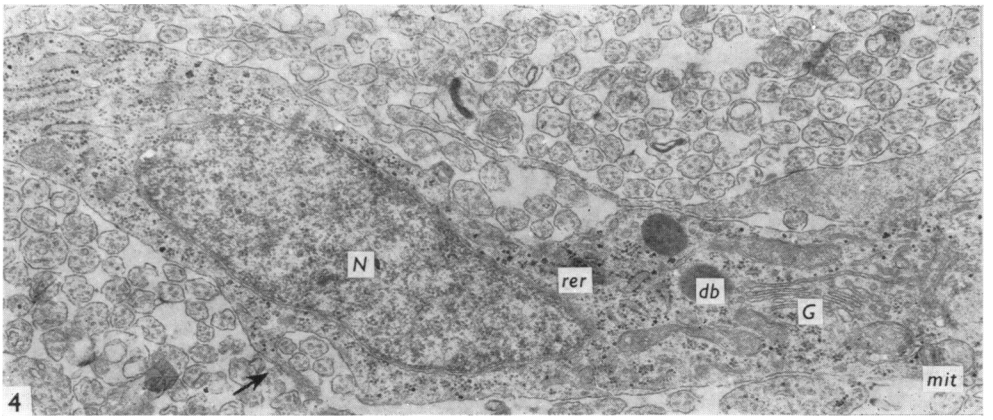
The glioblasts (Fig. 3) had an elongated or irregularly-shaped nucleus whose chromatin was finely granular, with occasional clumps adjacent to the nuclear membrane. The perikaryon was usually limited to a narrow rim around the nucleus: it contained a number of scattered free ribosomes. The processes contained rosettes of free ribosomes, short cisternae of rough endoplasmic reticulum filled with an

Fig. 3. 10 week astrocytic precursor cell. Note the narrow rim of perinuclear cytoplasm and the broad process containing scattered rosettes of free ribosomes, a few cisternae of rough endoplasmic reticulum, an extensive Golgi complex (*G*), a centriole (single arrow), and a cilium (double arrows). ×20000.

Fig. 4. 12 week early astrocyte. This cell has a fairly extensive cytoplasm containing scattered rosettes of free ribosomes, a number of short cisternae of rough endoplasmic reticulum (*rer*), dense bodies (*db*), a Golgi complex (*G*), and a few glycogen granules. Mitochondria (mit) are found much more frequently at this age than in younger embryos. The arrow shows a thin process partially enclosing four axons. *N*=nucleus. ×20000.

Fig. 5. 15 weeks post-conception. This shows a mitotic astrocyte in anaphase (*M*) containing glycogen granules (*gn*) and masses of microfibrils (*mf*), adjacent to two interphase astrocytes (A 1, A 2). ×20000.


G
3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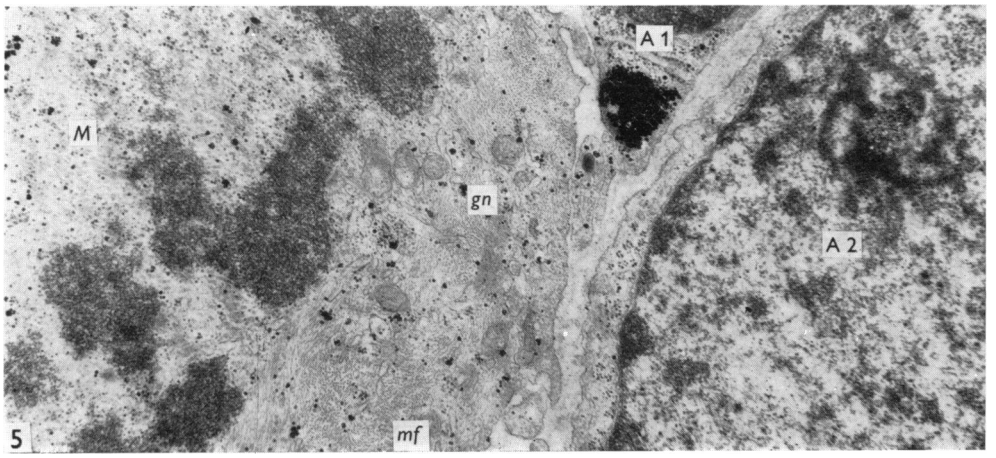

A 1
M
gn
A 2
mf
5

electron-dense homogeneous material, a Golgi complex and occasional dense bodies. Very few mitochondria were present, but cilia and centrioles were common (Fig. 3).

Astroblasts. Astroblasts were first observed at 10 weeks post-conception and were the most commonly found cells at 12 weeks.

The nuclei of astroblasts tended to be smaller than those of glioblasts. The perikaryon was more extensive (Fig. 4) and contained numerous rosettes of free ribosomes. The processes contained numerous mitochondria, an extensive Golgi complex, and a few dense bodies. The cisternae of rough endoplasmic reticulum were filled with a homogeneous substance and were present mainly in the cell processes. Small glycogen granules were sparsely scattered throughout the cytoplasm.

Astrocytes. With the exception of endothelial cells and pericytes all the cells found at 14, 15 and 18 weeks post-conception had the characteristics of astrocytes.

These cells contained glycogen granules and microfibrils. The nuclei varied in shape and irregular nuclei were common (Figs. 6, 8 and 9). The nucleus had a dense rim of chromatin with occasional clumps scattered throughout.

The perikaryon was usually restricted to a narrow rim of cytoplasm containing rosettes of free ribosomes and occasional cisternae of rough endoplasmic reticulum. The processes, broad at first, but soon narrowing, contained mitochondria, and long cisternae of rough endoplasmic reticulum, often arranged in parallel rows. Small dense bodies were common (Figs. 6, 8 and 9.)

A centriole and a single cilium were observed in many cells (Figs. 6–8), usually closely related to the extensive Golgi complex. From the appearance of the cross-sectioned cilium in Fig. 17 these cilia were probably not of the '9+2' variety but rather 'primary' cilia (Sorokin, 1968). Clumps of glycogen granules were scattered throughout the cytoplasm and often appeared to be particularly numerous where processes were in contact with other processes or perikarya (Fig. 9). Microtubules were occasionally present in the perikaryon (Fig. 8) but loose bundles of microfibrils were the most prominent feature in the processes.

Slender outgrowths from astrocytic processes were occasionally seen partially surrounding small groups of axons (Figs. 7 and 9).

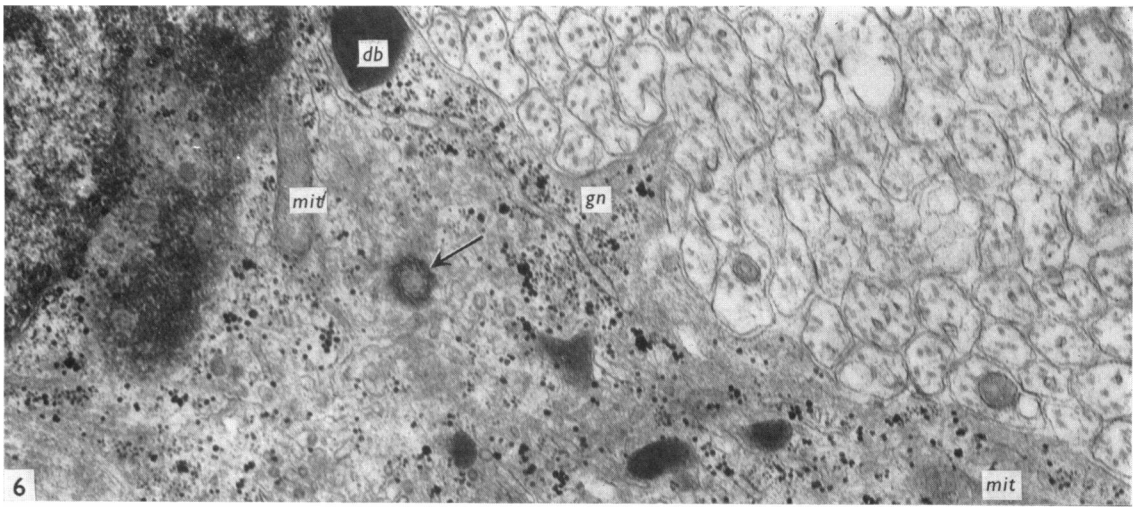
Astrocytes in the 18 week optic nerve tended to have a sparser cytoplasm, more densely packed with microfibrils (Fig. 10). The processes were more branched and contained few organelles apart from microfibrils, in contrast to the processes in younger embryos which usually contained a fair amount of organelles.

Mitotic cells. Despite the frequency of mitoses seen with the light microscope, few mitotic cells were observed with the electron microscope. Those found at 15 weeks were mature astrocytes. Figure 5 shows an anaphase containing scattered glycogen granules and extremely numerous microfibrils.

Fig. 6. 15 week astrocyte. This cell contains a large dense body (*db*), glycogen granules (*gn*), mitochondria (*mit*) and a centriole (arrow). ×30000.

Fig. 7. 15 week astrocyte with an extensive Golgi complex (*G*), glycogen granules, microfibrils (*mf*) and a transversely sectioned cilium. The cilium is probably in an extracellular trench. ×30000.

Fig. 8. 15 weeks post-conception. This shows a pair of astrocytes. The lower astrocyte has numerous glycogen granules, an extensive Golgi complex (*G*), a basal body of a cilium (single arrow) and a few microtubules (double arrows). ×30000.



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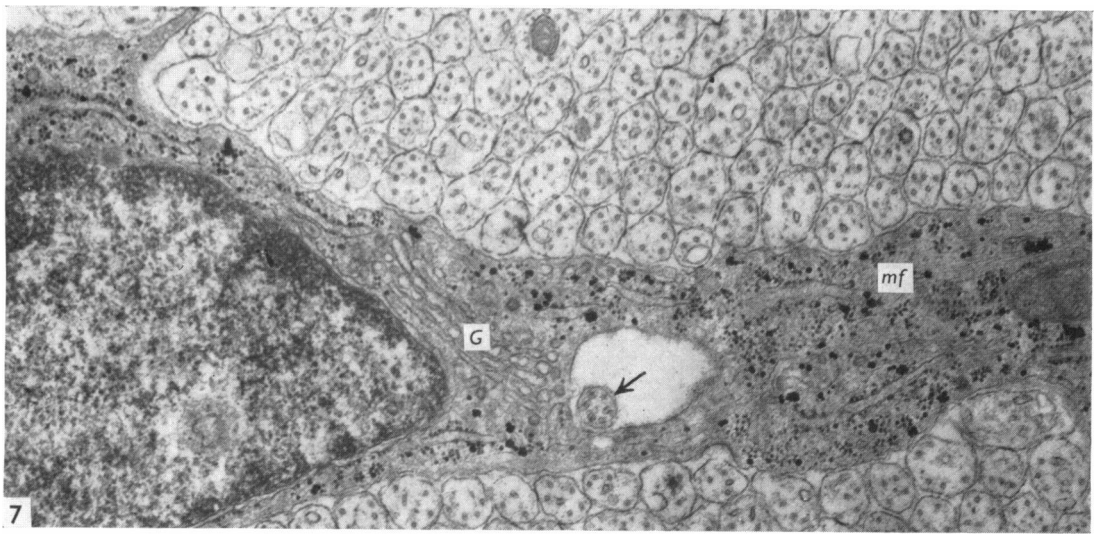

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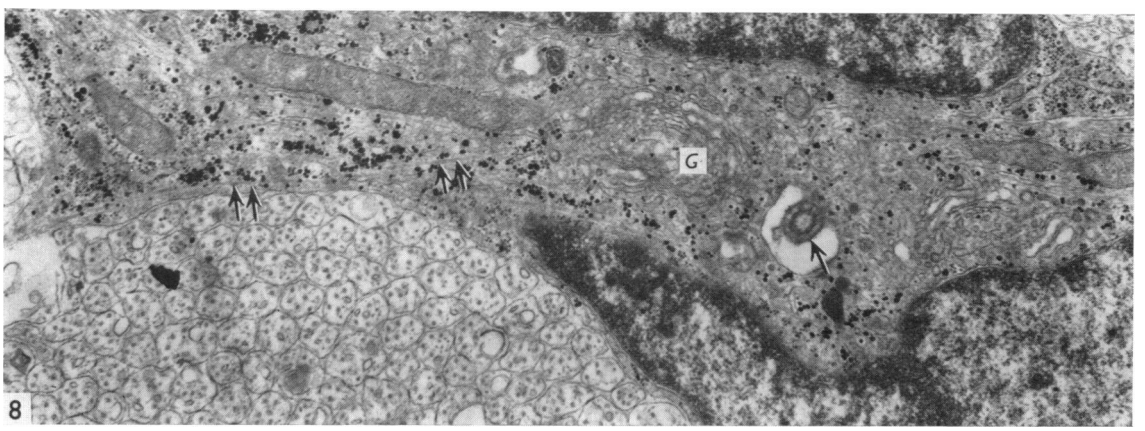

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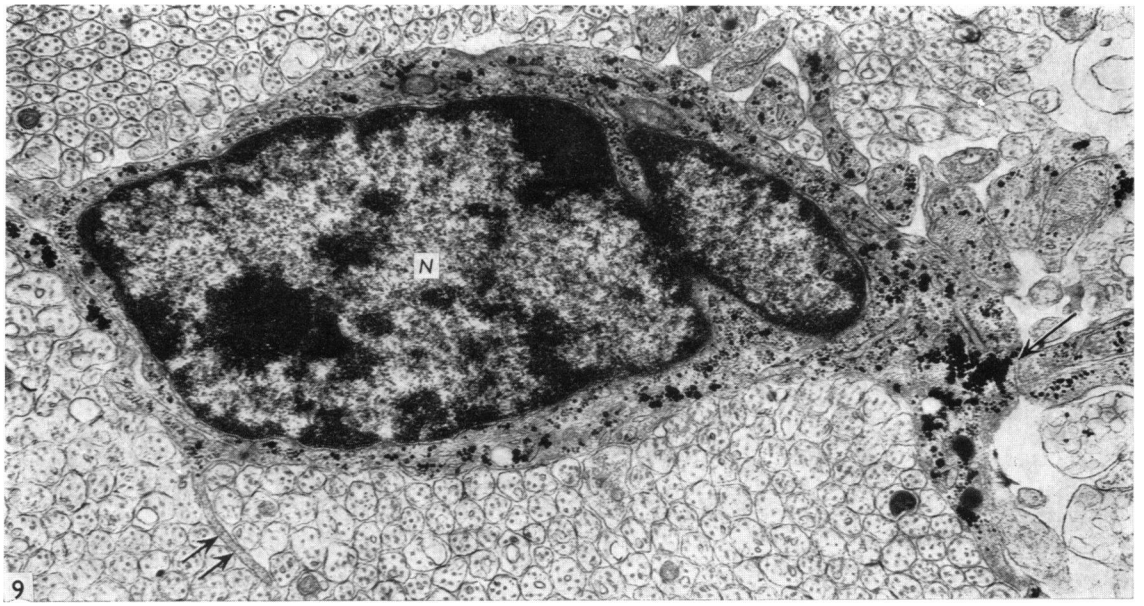

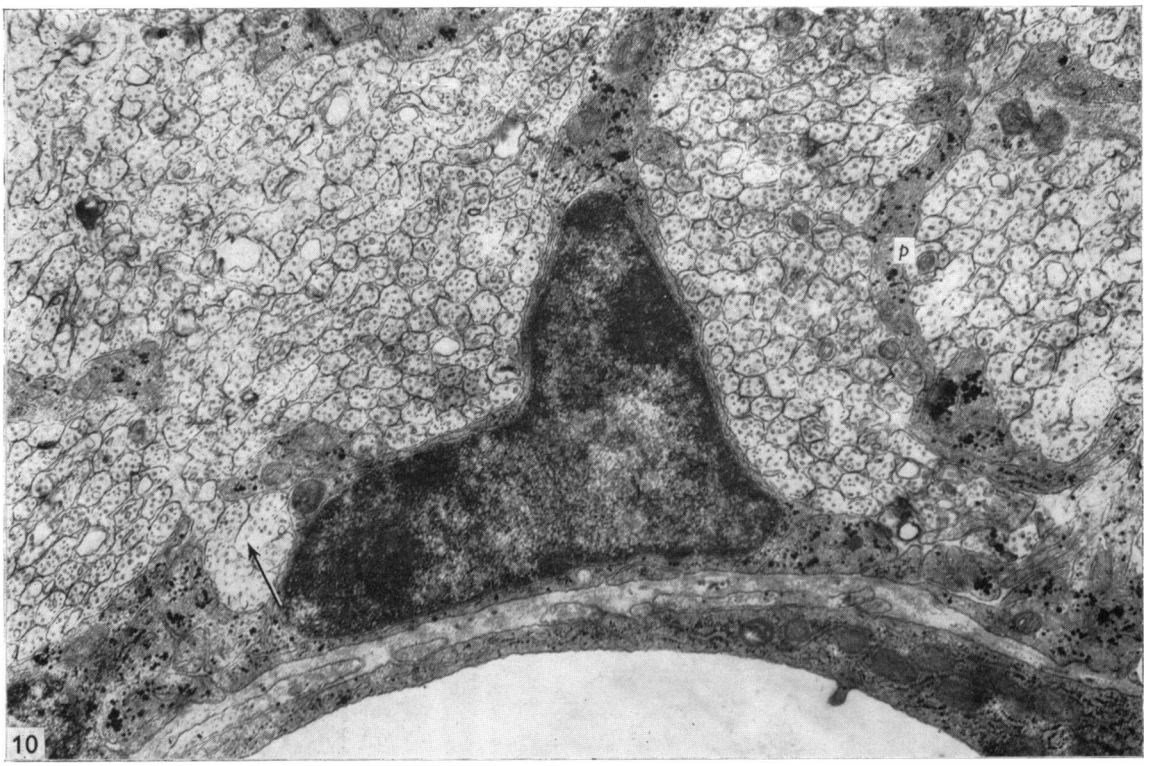

Fig. 9. 15 weeks post-conception. An astrocyte shows clumping of glycogen granules within the perikaryon (arrow) where another astrocytic process is in contact with it. Note the irregular nucleus (*N*). The double arrows indicate a slender process partly enclosing a group of axons. × 20000.

Fig. 10. 18 weeks post-conception. Perivascular astrocyte with narrow processes. Note the narrow astrocytic process (*p*) packed with microfibrils and containing a few scattered glycogen granules. The arrow shows a few axons almost totally enclosed by astrocytic processes. × 20000.

DISCUSSION

From 8 weeks post-conception the optic nerve can be seen to be fasciculated by cell processes. With the exception of vascular pericytes, all glial cells examined in the 18 week human embryonic optic nerve contained microfibrils and glycogen granules which were only present together in astrocytes (e.g. Maxwell & Kruger, 1965; Mori & Leblond 1969; Peters, Palay & Webster, 1970). Therefore all fasciculating cells at this age were astrocytes, and similar fasciculating cells observed in younger embryos which lacked these astrocytic characteristics were almost certainly astrocytic precursors. By assembling these cells in sequence, according to the time of their appearance, the maturation process of the astrocyte series may be visualised.

The earliest cells contained neither glycogen granules nor microfibrils. The characteristic feature of these cells was the dark cytoplasmic matrix, numerous free ribosomes and the frequent presence of cilia. These cells seemed similar to the dark glioblasts previously described in the developing mouse anterior commissure (Sturrock, 1974).

At 10 weeks processes could occasionally be seen which contained glycogen granules and microfibrils. By 12 weeks the amount of cytoplasm had increased, and organelles, particularly mitochondria, were more numerous. Glygocen granules were found in the majority of cells but in smaller quantities than at later stages.

At 14–15 weeks microfibrils were present in the perikaryon, and particularly in the cell processes. Glycogen granules were common, often forming clumps where processes were in contact with one another, or with the perikaryon of another cell.

While a few cells had differentiated into fairly mature astrocytes at 10 weeks, cells lacking both glycogen granules and microfibrils were still quite numerous at 12 weeks. By 14 weeks, however, all fasciculating cells contained both glycogen granules and microfibrils. Cilia, which were common in dark glioblasts, were also common in young astrocytes. It seems unlikely that such cilia have a motile function, and Barnes (1961) has suggested that they may be sensory. Similar cilia have also been found in astrocytoblasts of the kitten optic nerve (Blunt – personal communication).

By 18 weeks the processes had become thinner and contained few organelles apart from microfibrils, unlike the processes of young astrocytes, which contained numerous mitochondria and cisternae of rough endoplasmic reticulum. Glycogen granules were also much sparser in the processes, although glycogen clusters were occasionally found at points of contact.

Unlike the developing astrocytes in the rat optic nerve (Vaughn & Peters, 1967; Peters & Vaughn, 1967), the human astrocytic precursors contained few microtubules prior to the appearance of microfibrils. The absence of microtubules in developing astrocytes in the monkey spinal cord was noted by Phillips (1973). Microtubules are also infrequent in developing astrocytes in the mouse anterior commissure (Sturrock, 1974), and the suggestion of Peters & Vaughn (1967) that microtubules give rise to microfibrils appears unlikely on numerical grounds. Physical and chemical evidence (Davison & Huneeus, 1970; Wuerker & Kirkpatrick, 1972) suggests that there is in fact no relationship between microtubules and microfibrils.

The loose packing of axons in early embryonic optic nerves may be an artefact resulting from the absence of microfibrils in the astrocytic precursors which fasciculate the optic nerve at this stage, so making the nerve more liable to fraying during removal and processing; also the amount of fasciculation increases with age. Similar extracellular spaces were observed during development of the kitten optic nerve (Blunt, Baldwin & Wendell-Smith, 1972), and in the developing rat cerebrum (Caley & Maxwell, 1968). Blunt, Baldwin & Wendell-Smith (1972) suggested that this extracellular space served to allow further axonal growth and myelination, but in the human fetus the extracellular space has largely disappeared by 15 weeks post-conception (Fig. 7), although the onset of myelination does not take place until 36 weeks post-conception (Lucas Keene & Hewer, 1931; Langworthy, 1933). Furthermore, no such extracellular space was present in the developing anterior commissure of fetal mice (Sturrock, 1974).

The appearance of glycogen granules and mitochondria in astrocytes and their precursors during the increase in vascularization suggests that either glycogen synthesis occurs with increasing vascularization, or else that, prior to this time, glycogen exists in a different form. The large quantity of glycogen found from 14 weeks onwards suggests that these cells, as well as providing mechanical support, have a metabolic supporting role in the developing optic nerve.

The absence of other types of glial cells at 18 weeks suggests that either astrocytes de-differentiate and change into oligodendrocytes, or, more likely, that prior to myelination a further series of precursors giving rise to oligodendrocytes is produced. Vaughn (1969) showed that in the newborn rat optic nerve around 70% of the total glial population were astrocytes, and that oligodendrocytes increased in number just before and during myelination.

The number of astrocytes seemed to be related to the vascularity of the nerve. Although the mitotic index was low, it was probably sufficient to account for the increase in cell numbers, even taking into account that, as well as increasing in girth, the optic nerve was increasing in length at the same time.

As early as 15 weeks post-conception all the mitotic cells observed in the electron microscope, excluding endothelial cells, had the characteristics of mature astrocytes. This certainly suggests that the scheme of gliogenesis proposed by Vaughn (1969) for the rat optic nerve is not applicable to the human optic nerve, and at no time were cells similar to Vaughn's (1969) small glioblasts observed.

The presence of processes fasciculating the optic nerve in all embryos examined suggests that the processes of both astrocytes and astrocytic precursors must be sheet-like, rather than cylindrical, otherwise it is unlikely that they could have been followed for such distances in the nerve both by light and electron microscopy.

The large numbers of mature astrocytes found in the human optic nerve before 20 weeks of gestation must cast some doubt on the statement of Dobbing & Sands (1970) that the phase of multiplication in human brain from 15–20 weeks is purely neuroblastic. It seems probable that this phase also contains a considerable astrocytic element. The second phase described by these authors is probably oligodendrocytic and associated with myelination.

SUMMARY

Optic nerves from perfusion-fixed human embryos of 28, 50, 75, 105, 120 and 165 mm crown–rump length were examined in the electron microscope.

The number of glial cells per section was found to increase steadily from 10 weeks post-conception to 18 weeks and a close correlation ($r = 0{\cdot}92$) was found between the percentage vascularity and the glial population. Mitotic figures were present in all optic nerves examined.

From 14 weeks onwards all glial cells, except pericytes, were found to be fibrous astrocytes.

The human fibrous astrocyte appears to pass through the following stages of development: (1) Astrocytic precursors (dark glioblasts) have a dense cytoplasmic matrix with few organelles, although a single cilium is frequently present. (2) Concomitant with the increase in vascularization of the optic nerve found between 12 and 14 weeks glycogen granules increase in the cytoplasm of astrocytic precursors, followed by microfibrils, which appear first in the processes and later extend into the perikaryon. (3) With the appearance of glycogen granules the cytoplasmic organelles, particularly mitochondria, increase in amount and the cytoplasmic matrix gradually becomes less dense. (4) With increasing age fewer organelles are found in astrocytic processes, which become thinner and densely packed with microfibrils.

I should like to acknowledge the invaluable assistance of Mrs E. Lloyd-Davies in the preparation of material for electron microscopy. The human embryonic material was supplied by the Dundee University Department of Obstetrics and Gynaecology. Dr I. H. M. Smart kindly read the manuscript and provided advice and encouragement during the period of this study.

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